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APPLICATION FOR UNITED STATES LETTERS PATENT

SPECIFICATION

Be it known that we, Kari Alitalo, A Citizen of Finland, Research Professor of the Finnish Academy of Sciences, Molecular/Cancer Biology Laboratory, Haartman Institute, University of Helsinki, POB 21 (Haartmaninkatu 3), 00014 Helsinki, FINLAND; Erkki Koivunen, A Citizen of Finland, Department of Biosciences, Division of Biochemistry, Viikinkaari 5, FIN-00014 Helsinki, FINLAND; and, Hajime Kubo, A Citizen of Japan, Molecular/Cancer Biology Laboratory, Haartman Institute, University of Helsinki, POB 21 (Haartmaninkatu 3), SF-00014 Helsinki, FINLAND have invented a new and useful VEGFR-3 INHIBITOR MATERIALS AND METHODS, of which the following is a specification.

VEGFR-3 INHIBITOR MATERIALS AND METHODS

The present application claims priority to United States provisional application entitled "VEGFR-3 INHIBITOR MATERIALS AND METHODS" filed on January 17, 2001 and given the serial number 60/262,476. The entire text of the aforementioned disclosure is incorporated herein by reference without prejudice or disclaimer.

Field of the Invention

The present invention relates to the characterization and inhibition of VEGFR-3-mediated biological activities, especially those activities which are mediated by VEGFR-3 ligands VEGF-C and/or VEGF-D. More particularly, the present invention identifies peptide inhibitors of VEGF-C or VEGF-D binding to VEGFR-3. These inhibitors may be used in methods and compositions for ameliorating the effects of VEGF-C in various disorders, as well as screening and imaging of VEGFR-3 for purposes such as research and diagnosis.

Background of the invention

Cancer is still a major cause of death in the world at the beginning of the 21st century and remains a major focus for ongoing research and development. In recent years a promising approach to the therapeutic intervention of cancer has focused on antiangiogenesis therapies. This approach to intervening in cancer progression takes advantage of the idea that inhibiting or otherwise limiting the blood supply to tumors will deplete the tumor of oxygen and nutrients and will cause arrest of tumor cell growth and proliferation. This approach has been found to be effective and there are presently over 20 anti-angiogenic drugs undergoing various stages of evaluation in phase I, II or III clinical trials and numerous others in preclinical development.

While there are many different forms of cancer exhibiting a wide variety of properties, one factor which many cancers share is that, in order to be fatal, they must metastasize. Until such time as metastasis occurs, a tumor, although

it may be malignant, is confined to one area of the body. This may cause discomfort and/or pain, or even lead to more serious problems, nevertheless if it can be located prior to metastasis, the cancer may be managed or even removed by surgical intervention. So long as the residual cancer cells are kept in check, such a discrete cancer may be controlled without significant problems. However, metastasis will cause the cancerous cells to invaded the body and while surgical resection may remove the primary tumor, the metastatic spread of the cancer to disparate sites is very difficult to manage.

Metastasis to regional lymph nodes via lymphatic vessels is a common step in the progression of cancer. Metastasis is an important prognostic factor in many types of cancer and forms the basis for surgical and radiation treatment of local lymph nodes. The process of tumor metastasis is a multistage event involving local invasion and destruction of intercellular matrix, intravasation into blood vessels, lymphatics or other channels of transport, survival in the circulation, extravasation out of the vessels in the secondary site and growth in the new location (Fidler, et al., Adv. Cancer Res. 28, 149-250 (1978), Liotta, et al., Cancer Treatment Res. 40, 223-238 (1988), Nicolson, Biochim. Biophy. Acta 948, 175-224 (1988) and Zetter, N. Eng. J. Med. 322, 605-612 (1990)). Success in establishing metastatic deposits requires tumor cells to be able to accomplish these steps sequentially.

Recently, several lines of evidences indicate that lymphangiogenesis, the formation of lymphatic vessels, promotes lymphatic metastasis (Stacker et al., Nature Med. In press (2001); Skobe et al., Nature Med. In press (2001); Mandriota et al., Nature Med. In press (2001)]. The control of lymphangiogenesis may provide a new strategy for lymph node metastasis in cancer therapy. Up to date, however, no anti-lymphangiogenic agent is known to be in clinical development.

VEGF-C was recently identified as a growth factor for the lymphatic vascular system. See International Patent Application No. PCT/US98/01973, published as WO 98/33917 on August 6, 1998. One of its receptors, VEGFR-3, is expressed in all endothelial cells during early embryogenesis. During later stages of development, the expression of VEGFR-3 becomes restricted to lymphatic vessels

(Alitalo et al. US Patent Nos. 6,107,046 and 5,776,755; Joukov et al., EMBO J. 15, 290-298 (1996); Aprelikov et al., Cancer Res. 52, 746-748 (1992). VEGF-C stimulates lymphangiogenesis *in vivo*, and transgenic mice overexpressing VEGF-C in the skin are characterized by specific hyperplasia of the lymphatic network. Furthermore, VEGF-C, has also been shown to induce angiogenesis *in vitro* and *in vivo*. As VEGFR-3 was also reported to be up-regulated on tumor blood vessels, the present inventors suggest that signaling via VEGFR-3 may stimulate both tumor lymphangiogenesis and angiogenesis (International Patent Application No. PCT/US99/23525, published as WO 00/21560, incorporated herein by reference; Valtola et al., Am. J. Pathol. 154 1382-1390 (1999) Kubo et al., Blood 96, 546-553 (2000).

A large family of vascular endothelial growth factors have been identified which, together with their receptors, play important roles in both vasculogenesis and angiogenesis [Risau *et al., Dev Biol 125*:441-450 (1988); Zachary, *Intl J Biochem Cell Bio 30*:1169-1174 (1998); Neufeld *et al., FASEB J 13*:9-22 (1999); Ferrara, *J Mol Med 77*:527-543 (1999)]. Both processes depend on the tightly controlled endothelial cell proliferation, migration, differentiation, and survival. In addition to playing a key role in the progression of cancer, dysfunction of the endothelial cell regulatory system also is involved in several diseases associated with abnormal angiogenesis, such as proliferative retinopathies, age-related muscular degeneration, rheumatoid arthritis, and psoriasis.

In addition to VEGF-C (see *e.g.*, GenBank Acc. No. X94216; also known as VEGF related protein (VRP) or VEGF-2), the PDGF/VEGF family of growth factors also includes at least the following members: PDGF-A (see *e.g.*, GenBank Acc. No. X06374), PDGF-B (see *e.g.*, GenBank Acc. No. M12783), VEGF (see *e.g.*, GenBank Acc. No. Q16889 referred to herein for clarity as VEGF-A or by particular isoform), PlGF (see *e.g.*, GenBank Acc. No. X54936 placental growth factor), VEGF-B (see *e.g.*, GenBank Acc. No. U48801; also known as VEGF-related factor (VRF)), VEGF-D (also known as c-fos-induced growth factor (FIGF); see *e.g.*, Genbank Acc. No. AJ000185), VEGF-E (also known as NZ7 VEGF or OV NZ7; see *e.g.*, GenBank Acc. No. S67522), NZ2 VEGF (also known

as OV NZ2; see *e.g.*, GenBank Acc. No. S67520), D1701 VEGF-like protein (see e.g., GenBank Acc. No. AF106020; Meyer *et al., EMBO J 18*:363-374), and NZ10 VEGF-like protein (described in International Patent Application PCT/US99/25869) [Stacker and Achen, *Growth Factors 17*:1-11 (1999); Neufeld *et al., FASEB J 13*:9-22 (1999); Ferrara, *J Mol Med 77*:527-543 (1999)].

Members of the PDGF/VEGF family are characterized by a number of structural motifs including a conserved PDGF motif defined by the sequence: P-[PS]-C-V-X(3)-R-C-[GSTA]-G-C-C (SEQ ID NO: 1). The brackets indicate that this position within the polypeptide can be any one of the amino acids contained within the brackets. The number contained within the parentheses indicates the number of amino acids that separate the "V" and "R" residues. This conserved motif falls within a large domain of 70-150 amino acids defined in part by eight highly conserved cysteine residues that form inter- and intramolecular disulfide bonds. This domain forms a cysteine knot motif composed of two disulfide bonds which form a covalently linked ring structure between two adjacent ß strands, and a third disulfide bond that penetrates the ring [see for example, Fig 1 in Muller *et al., Structure 5*:1325-1338 (1997)], similar to that found in other cysteine knot growth factors, *e.g.*, transforming growth factor-β (TGF-ß). The amino acid sequence of all known PDGF/VEGF proteins, with the exception of VEGF-E, contains the PDGF domain. The PDGF/VEGF family proteins are predominantly secreted glycoproteins that form either disulfide-linked or non-covalently bound homo- or heterodimers whose subunits are arranged in an anti-parallel manner [Stacker and Achen, *Growth Factors 17*:1-11 (1999); Muller *et al., Structure 5*:1325-1338 (1997)].

The PDGF subfamily is reviewed in Heldin *et al., Biochimica et Biophysica Acta 1378*:F79-113 (1998).

The VEGF subfamily is composed of PDGF/VEGF members which share a VEGF homology domain (VHD) characterized by the sequence: C-X(22-24)-P-[PSR]-C-V-X(3)-R-C-[GSTA]-G-C-C-X(6)-C-X(32-41)-C (SEQ ID NO: 2-31). The VHD domain, determined through analysis of the VEGF subfamily members, comprises the PDGF motif but is more specific.

VEGF-C, comprises a VHD that is approximately 30% identical at the amino acid level to VEGF-A (discussed below). VEGF-C is originally expressed as a larger precursor protein, prepro-VEGF-C, having extensive amino- and carboxy-terminal peptide sequences flanking the VHD, with the C-terminal peptide containing tandemly repeated cysteine residues in a motif typical of Balbiani ring 3 protein. Prepro-VEGF-C undergoes extensive proteolytic maturation involving the successive cleavage of a signal peptide, the C-terminal pro-peptide, and the N-terminal pro-peptide. Secreted VEGF-C protein consists of a non-covalently-linked homodimer, in which each monomer contains the VHD. The intermediate forms of VEGF-C produced by partial proteolytic processing show increasing affinity for the VEGFR-3 receptor, and the mature protein is also able to bind to the VEGFR-2 receptor. [International Patent Publication No. WO 98/33917; Joukov et al., *EMBO J., 16:(13)*:3898-3911 (1997).] It has also been demonstrated that a mutant VEGF-C, in which a single cysteine at position 156 is either substituted by another amino acid or deleted, loses the ability to bind VEGFR-2 but remains capable of binding and activating VEGFR-3 [International Patent Publication No. WO 98/33917]. In mouse embryos, VEGF-C mRNA is expressed primarily in the allantois, jugular area, and the region of metanephros. [Joukov *et al., J Cell Physiol 173*:211-215 (1997)]. VEGF-C is involved in the regulation of lymphatic angiogenesis: when VEGF-C was overexpressed in the skin of transgenic mice, a hyperplastic lymphatic vessel network was observed, suggesting that VEGF-C induces lymphatic growth [Jeltsch *et al., Science, 276*:1423-1425 (1997)]. VEGF-C also shows angiogenic properties: it can stimulate migration of bovine capillary endothelial (BCE) cells in collagen and promote growth of human endothelial cells [see, *e.g.*, International Patent Publication No. WO 98/33917, incorporated herein by reference].

VEGF-D is structurally and functionally most closely related to VEGF-C [see International Patent Publ. No. WO 98/07832, incorporated herein by reference]. Like VEGF-C, VEGF-D is initially expressed as a prepro-peptide that undergoes N-terminal and C-terminal proteolytic processing, and forms non-covalently linked dimers. VEGF-D stimulates mitogenic responses in endothelial

cells *in vitro*. During embryogenesis, VEGF-D is expressed in a complex temporal and spatial pattern, and its expression persists in the heart, lung, and skeletal muscles in adults. Isolation of a biologically active fragment of VEGF-D designated VEGF-DΔNΔC, is described in International Patent Publication No. WO 98/07832, incorporated herein by reference. VEGF-DΔNΔC consists of amino acid residues 93 to 201 of VEGF-D linked to the affinity tag peptide FLAG®.

VEGF-A (also known as vascular permeability factor (VPF)), was originally purified on the basis of its mitogenic activity toward endothelial cells, and also its ability to induce microvascular permeability. Further information regarding the role of VEGF-A in various biological processes may be found in for example, Ferrara,(*J Mol Med 77*: 527-543, 1999); Neufeld *et al.*, (*FASEB J* 13: 9-22, 1999); Zachary, (*Intl J Biochem Cell Bio 30*: 1169-1174, 1998). VEGF-A is a secreted, disulfide-linked homodimeric glycoprotein composed of 23 kD subunits and five human isoforms have been described (Ferrara, *J Mol Med 77*:527-543; 1999); Neufeld *et al., FASEB J 13*:9-22; 1999). PlGF, a second member of the VEGF subfamily, is generally a poor stimulator of angiogenesis and endothelial cell proliferation in comparison to VEGF-A For additional information regarding this factor those of skill in the art are referred to, for example, Hauser *et al.*, (*Growth Factors 9*:259-268, 1993); Maglione *et al.*, (*Oncogene 8*:925-931, 1993) and Stacker and Achen, (*Growth Factors 17*:1-11; 1999).

VEGF-B, described in detail in International Patent Publication No. WO 96/26736 and U.S. Patents 5,840,693 and 5,607,918, shares approximately 44% amino acid identity with VEGF-A. VEGF-B has been shown to have angiogenic properties, and may also be involved in cell adhesion and migration, and in regulating the degradation of extracellular matrix. VEGF-B is expressed primarily in embryonic and adult cardiac and skeletal muscle tissues [Joukov *et al., J Cell Physiol 173*:211-215 (1997); Stacker and Achen, *Growth Factors 17*:1-11 (1999)]. Mice lacking VEGF-B survive but have smaller hearts, dysfunctional coronary vasculature, and exhibit impaired recovery from cardiac ischemia [Bellomo et al., Circ Res 2000;E29-E35].

Four additional members of the VEGF subfamily have been identified in poxviruses, which infect humans, sheep and goats [Ferrara, *J Mol Med 77*:527-543 (1999); Stacker and Achen, *Growth Factors 17*:1-11 (1999)]. VEGF-like proteins have also been identified from two additional strains of the orf virus, D1701 [GenBank Acc. No. AF106020; described in Meyer *et al., EMBO J 18*:363-374 (1999)] and NZ10 [described in International Patent Application PCT/US99/25869, incorporated herein by reference]. These viral VEGF-like proteins have been shown to bind VEGFR-2 present on host endothelium, and this binding is important for development of infection and viral induction of angiogenesis [Meyer *et al., EMBO J 18*:363-374 (1999); International Patent Application PCT/US99/25869].

The members of the PDGF/VEGF family are known to bind to cell surface receptors. Several such receptors have been identified, including for example, PDGFR-α (see *e.g.*, GenBank Acc. No. NM006206) , PDGFR-β (see *e.g.*, GenBank Acc. No. NM002609), VEGFR-1/Flt-1 (*fms*-like tyrosine kinase-1; GenBank Acc. No. X51602; De Vries *et al., Science 255*:989-991 (1992)); VEGFR-2/KDR/Flk-1 (kinase insert domain containing receptor/fetal liver kinase-1; GenBank Acc. Nos. X59397 (Flk-1) and L04947 (KDR); Terman *et al., Biochem Biophys Res Comm 187*:1579-1586 (1992); Matthews *et al., Proc Natl Acad Sci USA 88*:9026-9030 (1991)); VEGFR-3/Flt4 (*fms*-like tyrosine kinase 4; U.S. Patent No. 5,776,755 and GenBank Acc. No. X68203 and S66407; Pajusola *et al., Oncogene 9*:3545-3555 (1994)), neuropilin-1 (Gen Bank Acc. No. NM003873), and neuropilin-2 (Gen Bank Acc. No. NM003872). The two PDGF receptors mediate signaling of PDGFs as described above. $VEGF_{121}$, $VEGF_{165}$, VEGF-B, PlGF-1 and PlGF-2 bind VEGF-R1; $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, VEGF-C, VEGF-D, VEGF-E, and NZ2 VEGF bind VEGF-R2; VEGF-C and VEGF-D bind VEGFR-3; $VEGF_{165}$, PlGF-2, and NZ2 VEGF bind neuropilin-1; and $VEGF_{165}$ binds neuropilin-2.[Neufeld *et al., FASEB J 13*:9-22 (1999); Stacker and Achen, *Growth Factors 17*:1-11 (1999); Ortega *et al., Fron Biosci 4*:141-152 (1999); Zachary, *Intl J Biochem Cell Bio 30*:1169-1174 (1998); Petrova *et al., Exp Cell Res 253*:117-130 (1999)].

The PDGF receptors are protein tyrosine kinase receptors (PTKs) that contain five immunoglobulin-like loops in their extracellular domains. VEGFR-1, VEGFR-2, and VEGFR-3 comprise a subgroup of the PDGF subfamily of PTKs, distinguished by the presence of seven Ig domains in their extracellular domain and a split kinase domain in the cytoplasmic region. Both neuropilin-1 and neuropilin-2 are non-PTK VEGF receptors. NP-1 has an extracellular portion which includes a MAM domain; regions of homology to coagulation factors V and VIII, MFGPs and the DDR tyrosine kinase; and two CUB-like domains.

Several of the VEGF receptors are expressed as more than one isoform. A soluble isoform of VEGFR-1 lacking the seventh Ig-like loop, transmembrane domain, and the cytoplamic region is expressed in human umbilical vein endothelial cells. This VEGFR-1 isoform binds VEGF-A with high affinity and is capable of preventing VEGF-A-induced mitogenic responses [Ferrara, *J Mol Med 77*:527-543 (1999); Zachary, *Intl J Biochem Cell Bio 30*:1169-1174 (1998)]. A C-terminal truncated from of VEGFR-2 has also been reported [Zachary, *Intl J Biochem Cell Bio 30*:1169-1174 (1998)]. In humans, there are two isoforms of the VEGFR-3 protein which differ in the length of their C-terminal ends. Studies suggest that the longer isoform is responsible for most of the biological properties of VEGFR-3.

The receptors for the PDGFs, PDGF α-receptor (PDGFR-α) and the β-receptor (PDGFR-β), are expressed by many *in vitro* grown cell lines, and they are mainly expressed by mesenchymal cells *in vivo* (reviewed in [Raines et al., Peptide growth factors and their receptors, Heidelberg, Springer-Verlag (1990)]. As mentioned above, PDGF-B binds both PDGFRs, while PDGF-A selectively binds PDGFR-α.

Gene targeting studies in mice have revealed distinct physiological roles for the PDGF receptors despite the overlapping ligand specificities of the PDGFRs [Rosenkranz *et al., Growth Factors 16*:201-16 (1999)]. Homozygous null mutations for either of the two PDGF receptors are lethal. PDGFR-α deficient mice die during embryogenesis and show incomplete cephalic closure, impaired neural crest development, cardiovascular defects, skeletal defects, and odemas. The

PDGFR-β deficient mice develop similar phenotypes to animals deficient in PDGF-B, that are characterized by renal, hematological and cardiovascular abnormalities; where the renal and cardiovascular defects, at least in part, are due to the lack of proper recruitment of mural cells (vascular smooth muscle cells, pericytes or mesangial cells) to blood vessels.

The expression of VEGFR-1 occurs mainly in vascular endothelial cells, although some may be present on monocytes, trophoblast cells, and renal mesangial cells [Neufeld *et al., FASEB J 13*:9-22 (1999)]. VEGFR-1-/- mice die *in utero* between day 8.5 and 9.5. Although endothelial cells developed in these animals, the formation of functional blood vessels was severely impaired, suggesting that VEGFR-1 may be involved in cell-cell or cell-matrix interactions associated with cell migration. Recently, it has been demonstrated that mice expressing a mutated VEGFR-1 in which only the tyrosine kinase domain was missing show normal angiogenesis and survival, suggesting that the signaling capability of VEGFR-1 is not essential. [Hiratsuka et al., Proc. Natl Acad. Sci USA 95: 9349-9354 (1998); Neufeld *et al., FASEB J 13*:9-22 (1999); Ferrara, *J Mol Med 77*:527-543 (1999)].

VEGFR-2 expression is similar to that of VEGFR-1 in that it is broadly expressed in the vascular endothelium, but it is also present in hematopoietic stem cells, megakaryocytes, and retinal progenitor cells [Neufeld *et al., FASEB J 13*:9-22 (1999)]. Although the expression pattern of VEGFR-1 and VEGFR-2 overlap extensively, evidence suggests that, in most cell types, VEGFR-2 is the major receptor through which most of the VEGFs exert their biological activities. Examination of mouse embryos deficient in VEGFR-2 further indicate that this receptor is required for both endothelial cell differentiation and the development of hematopoietic cells [Joukov *et al., J Cell Physiol 173*:211-215 (1997)].

VEGFR-3, described in detail in U.S. Patent Nos. 5,776,755 and 6,107,046, incorporated here by reference, is expressed broadly in endothelial cells during early embryogenesis. During later stages of development, the expression of VEGFR-3 becomes restricted to developing lymphatic vessels [Kaipainen, A., *et*

al., Proc. Natl. Acad. Sci. USA, 92: 3566-3570 (1995)]. In adults, the lymphatic endothelia and some high endothelial venules express VEGFR-3, and increased expression occurs in lymphatic sinuses in metastatic lymph nodes and in lymphangioma. VEGFR-3 is also expressed in a subset of $CD34^+$ hematopoietic cells which may mediate the myelopoietic activity of VEGF-C demonstrated by overexpression studies [WO 98/33917]. Targeted disruption of the VEGFR-3 gene in mouse embryos leads to failure of the remodeling of the primary vascular network, and death after embryonic day 9.5 [Dumont et al., Science, 282: 946-949 (1998)]. These studies suggest an essential role for VEGFR-3 in the development of the embryonic vasculature, and also during lymphangiogenesis.

Structural analyses of the VEGF receptors indicate that the VEGF-A binding site on VEGFR-1 and VEGFR-2 is located in the second and third Ig-like loops. Similarly, the VEGF-C and VEGF-D binding sites on VEGFR-2 and VEGFR-3 are also contained within the second Ig-loop [Taipale *et al., Curr Top Microbiol Immunol 237*:85-96 (1999)]. The second Ig-like loop also confers ligand specificity as shown by domain swapping experiments [Ferrara, *J Mol Med 77*:527-543 (1999)]. Receptor-ligand studies indicate that dimers formed by the VEGF family proteins are capable of binding two VEGF receptor molecules, thereby dimerizing VEGF receptors. The fourth Ig-like domain on VEGFR-1, and also possibly on VEGFR-2, acts as the receptor dimerization domain that links two receptor molecules upon binding of the receptors to a ligand dimer [Ferrara, *J Mol Med 77*:527-543 (1999)]. Although the regions of VEGF-A that bind VEGFR-1 and VEGFR-2 overlap to a large extent, studies have revealed two separate domains within VEGF-A that interact with either VEGFR-1 or VEGFR-2, as well as specific amino acid residues within these domains that are critical for ligand-receptor interactions. Mutations within either VEGF receptor-specific domain that specifically prevent binding to one particular VEGF receptor have also been recovered [Neufeld *et al., FASEB J 13*:9-22 (1999)].

VEGFR-1 and VEGFR-2 are structurally similar, share common ligands ($VEGF_{121}$ and $VEGF_{165}$), and exhibit similar expression patterns during development. However, the signals mediated through VEGFR-1 and VEGFR-2 by

the same ligand appear to be slightly different. VEGFR-2 has been shown to undergo autophosphorylation in response to VEGF-A, but phosphorylation of VEGFR-1 under identical conditions was barely detectable. VEGFR-2 mediated signals cause striking changes in the morphology, actin reorganization, and membrane ruffling of porcine aortic endothelial cells recombinantly overexpressing this receptor. In these cells, VEGFR-2 also mediated ligand-induced chemotaxis and mitogenicity; whereas VEGFR-1-transfected cells lacked mitogenic responses to VEGF-A. Mutations in VEGF-A that disrupt binding to VEGFR-2 fail to induce proliferation of endothelial cells, whereas VEGF-A mutants that are deficient in binding VEGFR-1 are still capable of promoting endothelial proliferation. Similarly, VEGF stimulation of cells expressing only VEGFR-2 leads to a mitogenic response whereas comparable stimulation of cells expressing only VEGFR-1 also results in cell migration, but does not induce cell proliferation. In addition, phosphoproteins co-precipitating with VEGFR-1 and VEGFR-2 are distinct, suggesting that different signaling molecules interact with receptor-specific intracellular sequences.

The emerging hypothesis is that the primary function of VEGFR-1 in angiogenesis may be to negatively regulate the activity of VEGF-A by binding it and thus preventing its interaction with VEGFR-2, whereas VEGFR-2 is thought to be the main transducer of VEGF-A signals in endothelial cells. In support of this hypothesis, mice deficient in VEGFR-1 die as embryos while mice expressing a VEGFR-1 receptor capable of binding VEGF-A but lacking the tyrosine kinase domain survive and do not exhibit abnormal embryonic development or angiogenesis. In addition, analyses of VEGF-A mutants that bind only VEGFR-2 show that they retain the ability to induce mitogenic responses in endothelial cells. However, VEGF-mediated migration of monocytes is dependent on VEGFR-1, indicating that signaling through this receptor is important for at least one biological function. In addition, the ability of VEGF-A to prevent the maturation of dendritic cells is also associated with VEGFR-1 signaling, suggesting that VEGFR-1 may function in cell types other than endothelial cells. [Ferrara, *J Mol Med 77*:527-543 (1999); Zachary, *Intl J Biochem Cell Bio 30*:1169-1174 (1998)].

Neuropilin-1 was originally cloned as a receptor for the collapsin/semaphorin family of proteins involved in axon guidance [Stacker and Achen, *Growth Factors 17*:1-11 (1999)]. It is expressed in both endothelia and specific subsets of neurons during embryogenesis, and it thought to be involved in coordinating the developing neuronal and vascular system. Although activation of neuropilin-1 does not appear to elicit biological responses in the absence of the VEGF family tyrosine-kinase receptors, the presence of neuropilins on cells leads to more efficient binding of $VEGF_{165}$ and VEGFR-2 mediated responses. [Neufeld *et al., FASEB J 13*:9-22 (1999)] Mice lacking neuropilin-1 show abnormalities in the developing embryonic cardiovascular system. [Neufeld *et al., FASEB J 13*:9-22 (1999)]

Neuropilin-2 was identified by expression cloning and is a collapsin/semaphorin receptor closely related to neuropilin-1. Neuropilin-2 is an isoform-specific VEGF receptor in that it only binds $VEGF_{165}$. Like neuropilin-1, neuropilin-2 is expressed in both endothelia and specific neurons, and is not predicted to function independently due to its relatively short intracellular domain. The function of neuropilin-2 in vascular development is unknown [Neufeld *et al., FASEB J 13*:9-22 (1999); WO 99/30157].

The discovery of VEGF-A as a key regulator of vascular development has spurred active research using VEGF-based therapeutic angiogenesis in cardiovascular medicine, as well as for treating diseases characterized by pathological angiogenesis with VEGF antagonists. Subsequent identification of additional VEGF family proteins and their roles in vascularization have also led to the development of therapies based on these growth factors [Ferrara and Alitalo, *Nature Med 5*:1359-1364 (1999)]. Animal studies of hindlimb ischemia, and myocardial ischemia using VEGF-A or VEGF-C, delivered by administration of recombinant protein or gene transfer using naked DNA or adenoviral vectors, implicate these molecules in promoting vascularization and increasing coronary blood flow.

Therapies based on inhibiting the activity of VEGF growth factors are being tested to treat disease states characterized by pathological angiogenesis.

VEGF expression is upregulated in many human tumors including primary breast cancer and gastric carcinoma. Studies in mice indicate that tumor-associated angiogenesis and growth of the tumor cells can be inhibited by treating the animals with monoclonal antibodies against VEGF-A. Further animal studies showed that expression of a dominant negative VEGFR-2 mutant that prevents signaling through this receptor, or administration of recombinant VEGFR-1 or VEGFR-2 mutants, which only contain the extracellular portion of these receptors, suppresses growth of several tumor cell lines. These encouraging results led to clinical trials using humanized high affinity monoclonal antibodies against VEGF-A (rhuMAb VEGF) as VEGF-A inhibitors. Phase II studies using rhuMAb VEGF to treat non-small cell lung carcinoma, colorectal carcinoma, breast, and renal cell carcinoma are currently ongoing. Compounds targeting inhibition of VEGF-A activity are also being tested for therapeutic uses in cancer patients: small molecule inhibitors of VEGF-A are in Phase II trials, and monoclonal antibodies against VEGFR-2 are entering clinical trials.

Retinopathy associated with diabetes mellitus, occlusion of central retinal vein or prematurity has been correlated with increased levels of VEGF-A. Animal studies using monoclonal antibodies against VEGF-A or soluble VEGFR-1 or VEGFR-2 mutants containing only the extracellular domain fused to immunoglobulin gFc domain show suppression of retinal angiogenesis. VEGF-A is also detected in age-related macular degeneration (AMD), and its expression is thought to be the cause of neovascularization in this disease. Intravitreal delivery of recombinant humanized anti-VEGF-A Fab antibody fragment or injection of 2'-fluoropyrimidine RNA oligonucleotide ligands (aptamers) to treat AMD are currently in clinical trials. Compounds that inhibit the activity of VEGF growth factors may also be used to treat other disease states involving abnormal angiogenesis. These include ischemic-reperfusion related brain edema and injury, conditions associated with ovarian hyperplasia and hypervascularity such as the polycystic ovary syndrome, endometriosis, and ovarian hyperstimulation syndrome [Ferrara and Alitalo, *Nature Med* 5:1359-1364 (1999)].